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Planetary Quarantine Unit
Phoenix Field Station Section
Ecological Investigations Program
National Communicable Disease Center
Public Health Service
U.S. Department of Health, Education, and Welfare
Phoenix, Arizona

CONTRIBUTORS:

Research Activity	Certification and Inspection	Sterility Control Laboratory
J. R. Puleo	N. J. Petersen	G. J. Tritz
J. H. Marshall	R. Graves	N. Fields
G. Oxborrow		B. Moore

Report submitted by:

M. S. Favero, Chief
Planetary Quarantine Unit

Report reviewed and forwarded by:

G. J. Love, Assistant Chief
Phoenix Field Station

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1. At the request of NASA Headquarters, two toroidal transformers were examined for both external and internal microbial contamination. These components had been estimated by the supplier to contain between 10^4 and 10^5 microorganisms per transformer at the time of manufacture. Preliminary studies revealed a much lower concentration. Of the viable microorganisms recovered, all (less than 10^2) were external contaminants. No viable internal contaminants were detected.

2. Studies on the enumeration of microbial contaminants on surfaces were continued. Tests were performed to determine the effect of temperature of the rinse fluid on recovery of naturally-occurring microbial contaminants and spores of Bacillus subtilis var. niger from stainless steel surfaces by ultrasonication.

   Three methods of inoculation were employed. One consisted of producing aerosols of spores of B. subtilis var. niger suspended in ethanol (95%) and allowing for settling on surfaces within a specially designed chamber. The others consisted of inoculating the entire surface of a stainless steel strip with 0.1 ml of either an ethanol or aqueous spore suspension. Strips exposed to aerosols were air-dried for 2 to 3 hours in a laminar flow clean bench. The strips inoculated by pipette were air-dried overnight in a laminar flow clean bench. To test for naturally occurring airborne microorganisms, strips were exposed to the environment for 4 weeks prior to assay. Results showed that optimum recovery of inoculated spores was obtained when the temperature of the peptone water was 4 C and the tank solution 25 C. The mean percent recovery and the average number of spores recovered, with one exception, were higher under these conditions than with peptone water and the tank solution at 25 C. In most cases the differences were considered significant (Table 1). In addition, the inoculation method appeared to influence recovery rates. Spores inoculated onto the strips by pipette were more difficult to remove than those inoculated in the form of an aerosol. This occurred with peptone water at 4 C and 25 C (Table 2). In two experiments concerned with the removal of naturally-occurring airborne microorganisms from strips a higher mean number of viable microorganisms was recovered in peptone water at 25 C than at 4 C. In one experiment the differences were significant (Table 3). Studies will be undertaken to determine whether this difference was due to physical or biological factors.

3. Studies on the recovery of sublethally-injured microorganisms were continued. Some investigators have reported higher recoveries of spores exposed to ethylene oxide (ETO) upon extended incubation. A series of experiments was performed to determine the extent and significance of this phenomenon. As reported earlier, increased recoveries of B. subtilis var. niger spores exposed to ETO were observed with trypticase soy agar (TSA) and trypticase soy broth (TSB) when the incubation period was extended from 2 to 5 days. However, no significant increase in colony counts or number of tubes showing visible growth occurred after this period. The total incubation time was 35 days. During this quarter, spores from three species of bacteria--B. cereus T, B. subtilis 5320, and Clostridium roseum--were exposed to 600 mg/L of ETO for varying periods of time. The length of exposure was designed to kill most but not all of

the spores. Spores were recovered from stainless steel strips in the standard manner. The rinse fluid was diluted decimally and portions either were inoculated into tubes of TSB or plated with TSA. Counts were performed after 1, 2, 3, 4, 7, and 14 days of incubation at 32 C. The results showed that there was no significant increase in colony counts or in the number of positive tubes after 3 days (Tables 4, 5, 6, 7, 8, and 9).

4. Studies were initiated to compare the efficiency of solid and liquid culture media for recovery of anaerobic spores. Spores of _C. roseum_ were enumerated by using TSB plus 1.5% agar and TSB. In the latter instance, five series of multiple tube dilutions were used. A series consisted of five tubes for each of three decimal dilutions of the stock spore suspension. Most probable numbers (MPN) for the multiple tube dilutions were computed from the _Standard Methods for the Examination of Water and Wastewater_, American Public Health Association, 1965. For the plate counts, five replicate pour plates were used. The results (Table 10) showed that there was no significant difference between the two enumeration systems. As expected, however, the precision of the plate counts was higher than that of the multiple tube dilutions. Further studies will be conducted next quarter in which emphasis will be placed on evaluation of various types of liquid and solid culture media for recovery of dry-heat-injured anaerobic spores.

5. The comparative rates of dry-heat inactivation for spores of mesophilic, psychrophilic and thermophilic bacteria were determined. Tests were conducted at 125 C in a silicone oil bath. _B. subtilis_ var. _niger_ represented the mesophile, _B. stearothermophilus_ the thermophile, and a species of the genus _Bacillus_ obtained from J. Larkin of Washington State University the psychrophile. TAM medium, supplemented with magnesium sulfate and calcium chloride was used for sporulation. Spore suspensions were inoculated onto stainless steel strips and placed under vacuum for 16 hours over silica gel. The plating media was trypticase soy agar. Incubation was for 48 hours at 32 C and 55 C for the mesophile and thermophile, respectively. The psychrophile was incubated for 5 days at 15 C. The mesophile had the most heat-resistant spore population and the psychrophile the least (Figure 1). Further studies will be performed using additional strains and, if possible, similar types of anaerobic spores.

A method was developed for separating naturally-occurring bacterial spores from soil and clean room dust without the use of culture media. Dry soil samples were incubated at 55 C for 2 days, suspended in 95% ethanol, ultrasonicated for 60 minutes and filtered through sterile cloth towels. No fungi or vegetative bacteria were detected after this treatment. Preliminary tests showed that this naturally-occurring spore population was twice as resistant to dry heat (oven) as spores of _B. subtilis_ var. _niger_. Physical protection of spores by small soil particles did not appear to account for this resistance. Additional studies will be conducted to determine if the dry heat resistance of naturally-occurring spores changes after culture on artificial media.

6. Personnel from the Phoenix Field Station Section travelled to Cape Kennedy to provide visual surveillance of Lunar Orbiter 6, Mission 3, from the time of final microbiological sampling until the aerodynamic shroud was in place.

   An addition to the Sterility Control Laboratory was designed and the floor plan prepared. Construction cost estimates have been made.

   The final draft of a handbook for certification of microbiological control facilities for spacecraft was prepared and submitted to the Planetary Quarantine Office. This document, to be designated NPC-3, provides general guidance along with specific instructions and survey forms for those individuals assigned responsibility for recommending certification of facilities and personnel involved in the control of microbial contamination of space hardware.

   A vertical laminar flow clean bench was installed in the Phoenix laboratory. This bench permits work involving toxic fumes to be conducted in a bioclean environment and will be used for experimental work on the recovery of organisms from model plastic systems and small spacecraft components.

7. At Cape Kennedy monitoring of microbial contamination was continued in Hangars AE, AO, and S, Surveyor sterilization and assembly laboratory, Surveyor fuel loading room, and in the Lunar Orbiter camera room. A similar study was initiated in the Lunar Orbiter fuel loading room (Table 11).

   Using the swab-rinse technique, levels of microbial contamination were determined on the surfaces of Lunar Orbiters 6, 7, and 3 (Table 12), and on the surface of Surveyor 3, the Surveyor 3 shroud, and the Surveyor 3 adapter (Table 13).

   All data pertaining to air and swab sampling collected since the laboratory began operation were put into a computer, and stored on magnetic tapes.

TABLE 1. EFFECTS OF VARYING THE TEMPERATURES OF PEPTONE WATER AND ULTRASONIC TANK SOLUTION ON RECOVERY OF SPORES OF BACILLUS SUBTILIS VAR. NIGER FROM STAINLESS STEEL STRIPS.

Temperature of peptone water	Temperature of tank solution	Method of inoculation	Average no. of spores recovered[1]	Probability factor	Average percent recovery	Probability factor
4 C	25 C	ethanol aerosol	72	>0.5	98	<0.001
25 C	25 C	ethanol aerosol	77		86	
4 C	25 C	water suspension	70	<0.05	87	<0.01
25 C	25 C	water suspension	53		72	
4 C	25 C	ethanol suspension	210	<0.02	82	<0.02
25 C	25 C	ethanol suspension	154		67	

[1] Each value based on the mean of 15 samples.

TABLE 2. COMPARISON OF DIFFERENT INOCULATION METHODS ON RECOVERY OF SPORES OF BACILLUS SUBTILIS VAR. NIGER FROM STAINLESS STEEL STRIPS.

Temperature of peptone water	Temperature of tank solution	Method of inoculation	Average percent recovery[1]	Probability factor
25 C	25 C	ethanol aerosol	86	<0.01
25 C	25 C	water suspension	72	
25 C	25 C	ethanol aerosol	86	<0.01
25 C	25 C	ethanol suspension	67	
25 C	25 C	water suspension	72	>0.4
25 C	25 C	ethanol suspension	67	
4 C	25 C	ethanol aerosol	98	<0.01
4 C	25 C	water suspension	87	
4 C	25 C	ethanol aerosol	98	<0.001
4 C	25 C	ethanol suspension	82	
4 C	25 C	water suspension	87	>0.2
4 C	25 C	ethanol suspension	82	

[1] Each value is the mean of 15 samples.

TABLE 3. EFFECTS OF VARYING THE TEMPERATURE OF PEPTONE WATER AND ULTRASONIC TANK SOLUTION ON RECOVERY OF NATURALLY OCCURRING MICROORGANISMS FROM STAINLESS STEEL STRIPS.

Experiment	Temperature of peptone water	Temperature of tank solution	Average number of microorganisms recovered[1]	Probability factor
A	4 C	25 C	1,030	< 0.01
	25 C	25 C	1,640	
B	4 C	25 C	977	> 0.05
	25 C	25 C	1,517	

[1] Each value is the mean of 26 samples.

TABLE 4. EFFECT OF EXTENDED INCUBATION ON SPORES OF _BACILLUS SUBTILIS_ 5230 EXPOSED TO 600 MG/L OF ETHYLENE OXIDE AND RECOVERED IN TRYPTICASE SOY AGAR.

Minutes of exposure	Dilution	Mean number[1] of visible colonies per plate after incubation at 32 C					
		No. of days 1	No. of days 2	No. of days 3	No. of days 4	No. of days 7	No. of days 14
75	none	1.8	27.8	36.3	39.0	39.5	39.8
	10^{-1}	0.3	1.7	3.0	3.3	3.9	4.0
none; controls	10^{-3}	57.5	64.0	64.0	64.0	64.0	64.0
	10^{-4}	4.0	6.5	7.0	8.0	8.0	8.0

[1] Each value is the mean colony count from 10 samples.

TABLE 5. EFFECT OF EXTENDED INCUBATION ON SPORES OF <u>BACILLUS SUBTILIS</u> 5230 EXPOSED TO 600 MG/L OF ETHYLENE OXIDE AND RECOVERED IN TRYPTICASE SOY BROTH.

Minutes of exposure	Dilution	No. of tubes	Number of tubes showing visible growth after incubation at 32 C					
			No. of days 1	No. of days 2	No. of days 3	No. of days 4	No. of days 7	No. of days 14
75	none	10	0	10				
	10^{-1}	10	0	10				
	10^{-2}	10	0	5	6	6	7	7
	10^{-3}	10	0	1	1	1	1	1
None; controls	10^{-4}	8	8					

TABLE 6. EFFECT OF EXTENDED INCUBATION ON SPORES OF *BACILLUS CEREUS* T EXPOSED TO 600 MG/L OF ETHYLENE OXIDE AND RECOVERED IN TRYPTICASE SOY AGAR.

Minutes of exposure	Dilution	Mean number[1] of visible colonies per plate after incubation at 32 C: No. of days 1	No. of days 2	No. of days 3	No. of days 4	No. of days 7	No. of days 14
45	none	4.6	11.6	11.7	11.7	11.8	11.8
	10^{-1}	0.5	1.4	1.4	1.4	1.4	1.4
None; controls	10^{-4}	156.5	157.5	158.0	158.0	158.0	158.0
	10^{-5}	13.5	13.5	13.5	13.5	13.5	13.5

1 Each value is the mean colony count from 10 samples.

TABLE 7. EFFECT OF EXTENDED INCUBATION ON SPORES OF BACILLUS CEREUS T EXPOSED TO 600 MG/L OF ETHYLENE OXIDE AND RECOVERED IN TRYPTICASE SOY BROTH.

Minutes of exposure	Dilution	No. of tubes	Number of tubes showing visible growth after incubation at 32 C					
			No. of days 1	No. of days 2	No. of days 3	No. of days 4	No. of days 7	No. of days 14
45	none	10	10					
	10^{-1}	10	1	10				
	10^{-2}	10	0	5	5	5	5	5
	10^{-3}	10	0	1	1	1	1	1
None; controls	10^{-4}	8	8					

TABLE 8. EFFECT OF EXTENDED INCUBATION ON SPORES OF CLOSTRIDIUM ROSEUM EXPOSED TO 600 MG/L OF ETHYLENE OXIDE AND RECOVERED IN TRYPTICASE SOY AGAR.

Minutes of exposure	Dilution	Mean number[1] of visible colonies per plate after incubation at 32 C					
		No. of days 1	No. of days 2	No. of days 3	No. of days 4	No. of days 7	No. of days 14
15	none	12.9	16.3	16.4	16.5	16.8	16.8
	10^{-1}	0.9	1.2	1.2	1.2	1.2	1.2
None; controls	10^{-3}	206.0	207.0	207.0	207.0	207.0	207.0
	10^{-4}	23.5	24.0	24.0	24.0	24.0	24.0

[1] Each value is the mean colony count from 10 samples.

TABLE 9. EFFECT OF EXTENDED INCUBATION ON SPORES OF <u>CLOSTRIDIUM ROSEUM</u> EXPOSED TO 600 MG./L OF ETHYLENE OXIDE AND RECOVERED IN TRYPTICASE SOY BROTH.

Minutes of exposure	Dilution	No. of tubes	Number of tubes showing visible growth after incubation at 32 C					
			No. of days 1	No. of days 2	No. of days 3	No. of days 4	No. of days 7	No. of days 14
15	none	10	10					
	10^{-1}	10	8	9	9	9	9	9
	10^{-2}	10	4	6	6	6	6	6
	10^{-3}	10	3	3	3	3	3	3
None; controls	10^{-4}	7	7					

TABLE 10. COMPARATIVE RECOVERY RATES OF *CLOSTRIDIUM ROSEUM* SPORES USING SOLID AND LIQUID MEDIA.

Enumeration system	Mean number per ml	Standard deviation	Probability factor[1]
Plate count[2]	23	2.5	>0.2
Multiple tube dilution[3]	28	8.5[4]	

[1] Probability of difference between the means occurring by chance alone.

[2] Trypticase soy broth (B.B.L.) plus 1.5% agar.

[3] Trypticase soy broth plus 0.075% agar. Each series consisted of 5 tubes for each of 3 dilutions.

[4] Based on the mean of the most probable number from each of 5 series of multiple tube dilutions.

TABLE 11. ACCUMULATION OF MICROORGANISMS ON STAINLESS STEEL STRIPS EXPOSED TO THE INTRAMURAL ENVIRONMENT OF THE LUNAR ORBITER FUEL LOADING ROOM.

Weeks of exposure	Mesophilic Microorganisms			
	Aerobes No./sq. ft.	Anaerobes No./sq. ft.	Aerobic spores No./sq. ft.	Anaerobic spores No./ sq. ft.
1	2,102	900	238	122
2	418	0	122	58
3	58	122	122	0
1[1]	482	0	302	0
2	360	180	482	360
3	1,344	778	958	360
4	1,260	360	540	122
5	4,082	180	720	418
6	13,615	1,562	778	720
7	2,880	302	900	58
8	4,860	--	662	--
9	4,198	662	3,542	302
10	3,478	1,138	662	58
1[1]	58	0	0	0
2	0	0	0	0
3	180	0	58	0

[1] New series of strips exposed

TABLE 12. LEVEL OF MICROBIAL CONTAMINATION ON THE SURFACE OF THREE LUNAR ORBITERS.

Spacecraft	Area Sampled[1] (sq. in.)	Date	Mesophilic Microorganisms			
			Aerobes	Anaerobes	Aerobic spores	Anaerobic spores
6	59.1	10-17-66	35	15	10	0
6	59.1	12-28-66	85	20	5	10
6	59.1	1-13-67	355	185	10	0
6	59.1	1-24-67	180	50	10	0
7	59.1	1-3-67	15	20	10	5
7	59.1	2-3-67	270	5	10	5
3	59.1	3-10-67	2,575	785	5	5

[1] Swab-rinse technique.

TABLE 13. LEVELS OF MICROBIAL CONTAMINATION ON THE SURFACE OF SURVEYOR 3, THE SURVEYOR 3 SHROUD, AND THE SURVEYOR 3 ADAPTER.

Part sampled	Area sampled[1] (sq. in.)	Date	Mesophilic Microorganisms			
			Aerobes	Anaerobes	Aerobic spores	Anaerobic spores
Spacecraft	80	2-16-67[2]	0	0	0	0
Spacecraft	80	2-27-67	605	60	0	0
Spacecraft	80	2-28-67	480	175	5	0
Spacecraft	80	3-2-67	1,320	245	0	5
Spacecraft	80	3-7-67	1,985	435	5	0
Spacecraft	80	3-10-67	1,140	35	0	5
Adapter[3]	40	3-2-67	5,330	3,535	10	0
Adapter	40	3-8-67	1,205	130	10	0
Shroud	40	3-2-67	2,375	90	0	0
Shroud	36	3-8-67	2,380	170	25	0

1 Swab-rinse technique.

2 The spacecraft was enclosed in a cannister during shipment to Cape Kennedy. Samples were taken immediately after removal from the cannister.

3 Support stand for the spacecraft.

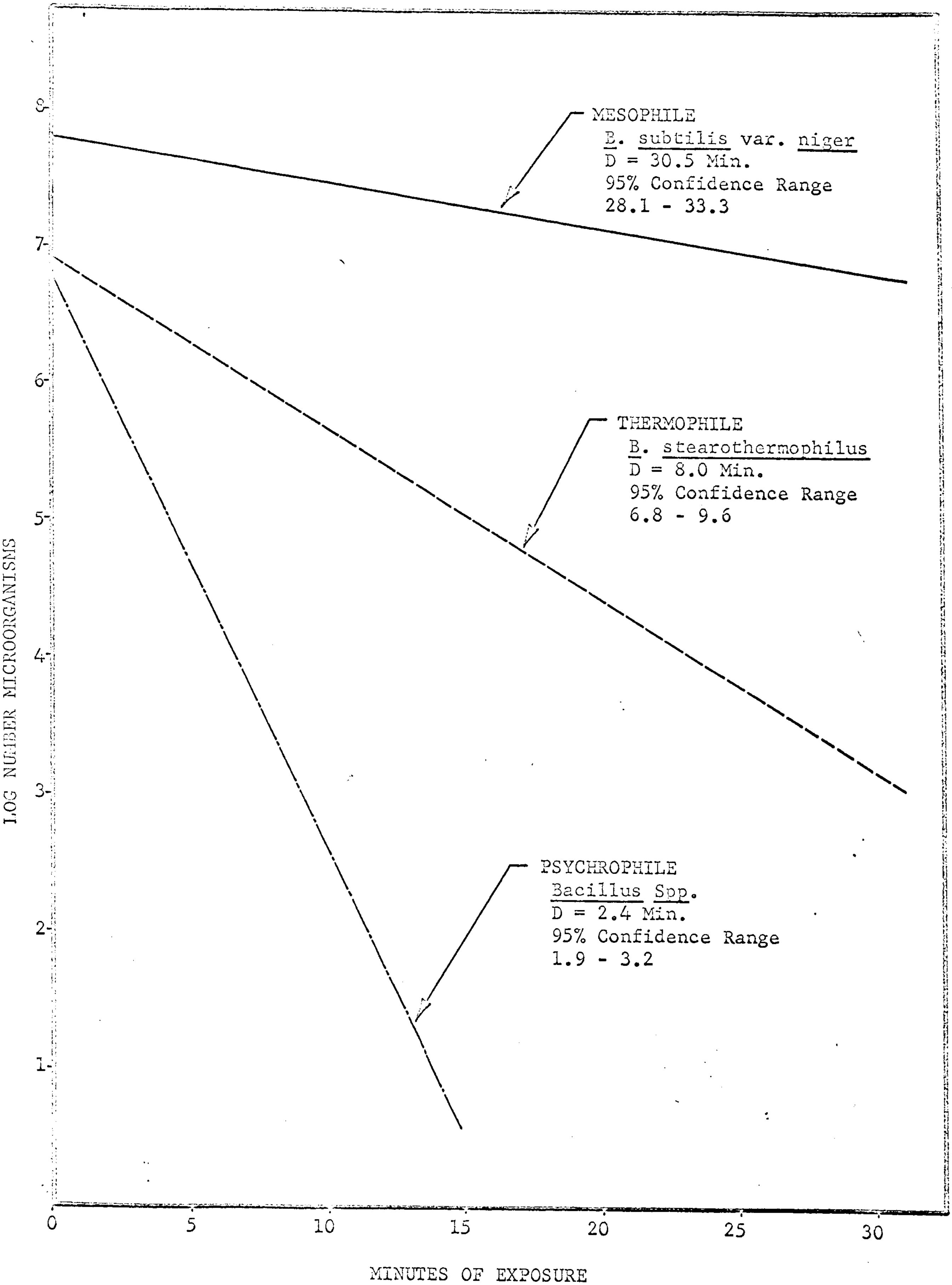

FIG. 1. Comparative rates of dry heat inactivation at 125° C. for spores of mesophilic, psychrophilic and thermophilic bacteria in the genus Bacillus.